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Determination of the Incubation Period of *Bacillus larvae* White, Causal Organism of American Foulbrood in Honey Bees (*Apis mellifera* L.)*

By O. W. Park

Diseases of the immature stages of the honey bee, i.e. the brood, cause large annual losses in bees, honey, and equipment. It would be bad enough if such losses were reflected only in the increased cost of producing honey, but far more serious is the decreased yield of at least 50 orchard, garden and farm crops. These are crops such as apples, cucumbers and the clovers which require or benefit from the pollinating services of the honey bee.

Two types of brood diseases, both known as foulbrood, had been recognized prior to 1900, but were inadequately distinguished until White (1907, 1912) determined their respective pathogens. Since one of these diseases had previously been under study in Europe, he called it *European,* and the other, *American* foulbrood.

For centuries the disease now known as American foulbrood has been the most serious scourge of the honey bee. Distribution is practically world-wide and no sizeable region in the United States is free from its depredations (Dadant, 1937). Although both foulbroods commonly produce disagreeable odors, the term *foulbrood,* doubtless came into use because of the outstanding and characteristically *foul* odor given off by American foulbrood. This probably is the malady which Aristotle (Cresswell translation, 1907) said, "causes a strong smell in the hives".

American foulbrood is caused by a specific spore-forming organism named *Bacillus larvae.* Its only known host is the honey-bee larva, so human beings are never affected. The disease is transmitted from larva to larva and from colony to colony by the spore stage only. The spores are extremely resistant to sunlight, desiccation, common disinfectants, and the germicidal action of honey. Freezing apparently has no deleterious effect, and spores have been known to survive boiling in water for as long as 5 hours, autoclaving at 15 pounds for 25 minutes, dry heat at 98° C. for 46 hours, and in melted beeswax at 100° C. have survived for nearly 5 days (Burnside, 1938). Maximum time spores remain virulent is not known

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but viable ones have been recovered from honey and from old brood combs after many years.

Spread of the disease from colony to colony (except when a bungling beekeeper inadvertently helps matters along) results largely because of the unbridled propensity of honey bees to rob their neighbors. Especially during dearth periods, when nectar is not available in the flowers, honey-bee foragers spend much time searching for inadequately protected booty. Stores of honey in a colony either dead or seriously weakened by American foulbrood seldom remain long undiscovered by other colonies, if there are any within a radius of 2 miles or so. Spores of *B. larvae* thus are carried home to be distributed by the nurse bees to the hungry larvae along with their food.

Spores ingested with contaminated food germinate in the stomach of a healthy larva that is less than 2 days old (Woodrow, 1941), giving rise to the rod-shaped vegetative stage. Upon death of the larva, which results from the growth and multiplication of the rods, the organism not only sporulates but also produces typical putrefaction. Death typically does not overtake the larva until after it has completed the feeding stage. By that time its cell has been capped over, the larva has spun its cocoon, and lies lengthwise in the cell, ready for its transformation into a pupa. In some cases, however, numerous larvae die before they are sealed and while still coiled. In other instances, development continues well into the pupal stage before death occurs.

Symptoms

Due to production by it of an antibiotic, *B. larvae* normally is the only organism associated with this disease. For this reason, in large part, the symptoms of American foulbrood are remarkably uniform. Thus, for instance, the foul odor produced is so uniform that it is looked upon as one of the more dependable characteristics to be considered in gross diagnosis.

The purpose of this paper, however, is not to rehearse well-established symptoms of American foulbrood, but rather to report on certain records pertaining to the duration of the incubation period of its causal organism, *Bacillus larvae*. These records were secured over the 10-year period, 1940-49, in connection with a research program, developed under the Iowa Agricultural Experiment Station (1935 to date), which has for its goal the development of strains of honey bees highly resistant or, if possible, immune to American foulbrood.

Before undertaking a program such as that just mentioned, it was essential to learn two basic facts: 1. Does variation in resistance to American foulbrood exist in honey bees? 2. In case a difference were found, could the character responsible for resistance be transmitted to offspring?

If the answer to either is "no", it would be useless to press the matter further. In case the answer to both should be "yes", there would be good reason to expect at least a measure of success in such a venture.

Briefly, our plans for determining whether variation in resistance exists were to obtain supposedly resistant stock from every possible source, bring such colonies together in one place, and inoculate them with uniform doses of the pathogen. These plans were carried out in 1935. Following inoculation, all six check colonies—presumably susceptible—took the disease promptly and showed no signs of recovery. Of 25 presumably resistant colonies, seven cleaned up all symptoms of the disease before fall.

Queens heading colonies that showed exceptional resistance in 1935 were used as breeders in 1936, with the result that one-third of the tested progeny recovered before the close of that season. Thus during the first two seasons, it was demonstrated that resistance to American foulbrood does exist in honey bees, that such resistance is heritable, and that eventual development of strains of honey bees highly resistant to this disease was well within the realm of possibility. Incidentally, it may be reported that, although gratifying progress has been made to date, further intensive testing, selection and breeding will be required before our ultimate objective is reached.

Against the foregoing background, let us consider some of the techniques employed in securing the records about to be presented.

Inoculations

Inoculations were made by removing a rectangle of comb from within the area occupied by brood in a healthy colony, and replacing it with a similar rectangle cut from an infected comb containing numerous American foulbrood "scales", which are the desiccated remains of bee larvae dead of the disease. Rectangles used were about 2 by 2½ inches, and contained about 100 scales, with 75 as a minimum. This method rarely failed to produce the disease except in a few of the most highly resistant colonies.

Diagnosis

Diagnoses made during the early years of this investigation usually were based on observation of gross symptoms, backed up when necessary by laboratory diagnosis for those instances in which only doubtful specimens were found by a thorough-going inspection of all brood in the colony. Laboratory diagnoses were made either by microscopic examination, cultural techniques or both. During the 10-year period covered by this report, these techniques were used extensively, partly as a means for improving our ability to correctly diagnose, in the field, specimens in which the disease had not yet progressed to the stage which exhibits its better-known gross diagnostic characters.

Another objective was to secure, if possible, postive evidence of the presence of the pathogen in *every* colony tested. This goal actually was attained in both 1943 and 1944, and was missed by only a very small percentage in most of the other seasons under consideration. In a few instances, however, it was necessary to administer a second or even a third inoculation before postive evidence of the pathogen could be obtained.

In a number of such cases, the dosage used for a second or third inoculation was stepped up by 7- to 50-fold. Of 14 such cases, a few cells of American foulbrood developed in seven, all of which recovered quickly. One colony developed considerable disease but by fall had reduced it from a peak of 350 cells to a low of 30 cells, and by June 25 of the following summer had completely recovered. All of the other six remained negative.

The season's total count of infected larvae in all 14 colonies was 872 *postives* and 252 *doubtfuls,* but if the two relatively high-count records (colonies 230 and 121) be omitted, said totals drop to the remarkably low level of 22 positives and 160 doubtfuls. This is an average of less than 2 postive and 12 doubtful cells per colony, based on an average of five inspections over a period of approximately 7 weeks subsequent to inoculation—an outstanding display of high-level resistance.

It would appear, therefore, that failure to recover the pathogen from a given colony subsequent to inoculation by our standard procedure is not to be interpreted as lack of adequate opportunity for the colony to become infected, but rather as an indication that said colony possesses to a high degree, the desired character for resistance to American foulbrood.

Field diagnosis, based on gross symptoms characteristic of the disease has in general been found practical and, for the most part,

rather adequate. And, in view of the fact that it is more practical to re-inoculate than to bother with laboratory techniques, current practice is to reduce the latter to a minimum.

Technique for microscopic examination for *B. larvae* was available from the beginning, and was employed as a useful check on field diagnoses based on the better-known gross characteristics. It was, however, unsatisfactory for detecting the presence of the vegetative stage—often the only stage present in specimens in which gross characters were inadequate for field diagnosis.

Facilities for laboratory diagnosis by cultural methods were, therefore, set up in 1937, and were used extensively during the period 1937 to 1940, inclusive, and again in 1945. During the other years of the 1940-49 period, cultural methods were employed only as required to supplement field diagnosis.

A cultural procedure devised by Lochhead (1927, 1928, 1933), and modified by Sturtevant (1932), was found adaptable to our needs. In this technique, a broth containinug peptone and carrot extract are used. The test depends on the ability of *B. larvae* to give a postive test for nitrite in carrot media containing no added nitrate. After incubation at 37° C. for 48 hours, the presence of *B. larvae,* even in minute amounts, causes the broth to change to a red color upon addition of sulphanilic acid and alphanaphthylamine.

Burnside (1940) showed that this test is not entirely specific for *B. larvae* but, as stated by Sturtevant (1932), "A postive nitrite test even in the absence of visible growth, is strong presumptive evidence of the presence of viable cells of *B. larvae.*" This method was especially helpful in the diagnosis of doubtful specimens of young larvae suspected of being in the very early stages of the disease.

Incubation Period

At the outset, inoculated colonies were inspected almost daily until the disease appeared. Early experience showed that symptoms suitable for field diagnosis seldom appeared before the 11th day. Further experience, supported by an ever-increasing number of records, showed that the highest percentage of positive diagnoses was obtained when inspections were made on the 12th or 13th day after inoculation.

In order, therefore, to conserve time, inspection schedules were set up for the 12th day, so that any overrun could be completed on the 13th day, with little or no loss of potential records. In any event failure to find postive evidence of the disease, at a regularly scheduled inspection, was followed by further inspections at 2- or 3-day

intervals until the disease was found, or until the likelihood of its belated appearance was considered to have passed. Judgment on this point of course had to be developed on the basis of experience.

Determination of the 928 time intervals involved in the accompanying tabulation, and also in the graphic presentation of the frequency distribution of said records, was independent of the method by means of which diagnosis was reach. As may be noted, the mean time lapse between inoculation and first postive diagnosis was found to be approximately 12.5 days. Yearly means show a considerable degree of uniformity, varying in all cases by less than 1 day from the overall mean. The dependability of this figure is further enhanced by the fact that, on the basis of frequency distribution of the time-lapse data, the modal class, containing nearly half of all the records, likewise falls on the 12th day, while the skewness of the curve would indicate that the modal value lies somewhere between the 12th and 13th days.

From the frequency-distribution chart, it may be further observed that only about 1 percent of the records fell on the 10th day or earlier, that barely 2 percent fell beyond the 16th day, and that only 5 percent fell beyond the 15th. On this basis, current practice is to re-inoculate all colonies from which substantial evidence of the pathogen has not been obtained within 16 days after inoculation.

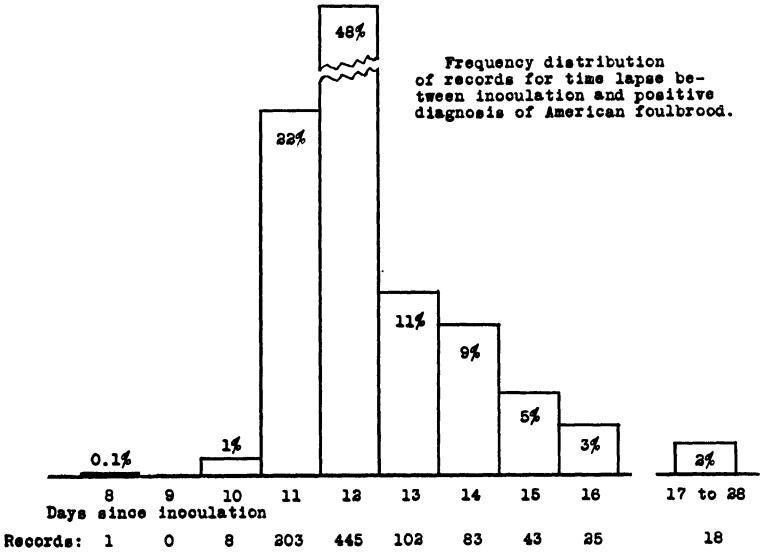
It is realized that since setting up our schedules for first inspections on the 12th or 13th day, there has been little opportunity to secure records that might have fallen short of a12- day period. But judging from the frequency distribution chart, it seems obvious that

Table 1

Time lapse between inoculation and first positive diagnosis of American foulbrood.

Year	Number of Inoculations	Total Days	Mean Days
1940	166	2,166	13.0
41	75	934	12.5
42	103	1,335	13.0
43	103	1,303	12.7
44	56	649	11.6
45	80	945	11.8
46	115	1,447	12.6
47	79	954	12.1
48	93	1,128	12.1
1949	58	719	12.4
10-Yr. Totals	928	11,580	123.8
10-Yr. Means	92.8	12.5	12.4±0.2

the data rather adequately cover the principal objectives of our case, which were: 1. To discover on which day following inoculation the greatest possible percentage of postive diagnoses could be secured, and 2. To find out how long after inoculation it is worthwhile to wait for the disease to appear, in any given case, before re-inoculating.



Discussion

From the foregoing, it will be understood that the term "incubation period" is used herein in the same practical sense it is used by the practicing physician in reply to a mother's question, "How long after exposure to measles before Susie will break out?" We find no fault with those who use the term otherwise, but deem it desirable to point out the need for realizing that such differences exist, when considering comparable results of other writers.

Sturtevant (1924) states, "The incubation period of *Bacillus larvae* is 24 to 48 hours." His interpretation is, "Germination of spores and some growth take place during the first 24 hours' incubation at 37° C., but maximum growth is not obtained much before 48 hours." And he explains further that growth sufficient to kill the larva does not occur until on or after the 8th day. Through conversation with Dr. Sturtevant, some years later, it was learned that he had only rarely found specimens that could be diagnosed satisfactorily in the field earlier than about the 10th or 11th day after having fed spores in sirup.

While agreeing with White (1920), that the first symptoms of American foulbrood may appear about the end of the first week after infection, we hasten to add that, in the light of our experience, symptoms which appear before about the 10th day seldom justify a positive diagnosis. Furthermore, with the comb-insert method of inoculation, it is possible that larvae may not become infected as soon as when spores are fed in sirup, although we consider the former technique definitely more reliable than the latter for producing infection in the colony.

With reference to laboratory cultures, Tarr (1937) reported: "When vegetative cells of *B. larvae* are required, these cultures are incubated not longer than 48 hours, while an incubation period of 1 week is employed when spores are required." And, subsequent to spraying young larvae with a spore suspension in water, he found that the ropy stage usually appeared in from 14 to 20 days. Apparently no inspection was made between the 10th and 14th days, so it seems possible this typical stage may have been present in some instances as early as the 11th or 12th day. On one occasion (1938), gross symptoms were observed on the 7th day, and by the 11th day the number of affected larvae had increased noticeably.

Figures just cited from other workers differ somewhat from those arrived at in the present study, but when due allowances are made for differences in objectives, terminology, and techniques, it is found that such discrepancies are relatively unimportant and that they are more apparent than real.

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